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Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) Gag- and Gag Peptide-Specific CD4$^+$ T-Cell Clones from an HIV-1-Seronegative Donor following In Vitro Immunization

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Substantial evidence argues that human immunodeficiency virus type 1 (HIV-1)-specific CD4$^+$ T cells play an important role in the control of HIV-1 replication in infected individuals. Moreover, it is increasingly clear that an HIV vaccine should elicit potent cytotoxic lymphocyte and antibody responses that will likely require an efficient CD4$^+$ T-cell response. Therefore, understanding and characterizing HIV-specific CD4$^+$ T-cell responses is an important aim. Here we describe the generation of HIV-1 Gag- and Gag peptide-specific CD4$^+$ T-cell clones from an HIV-1-seronegative donor by in vitro immunization with HIV-1 Gag peptides. The Gag peptides were able to induce a strong CD4$^+$ T-cell immune response in peripheral blood mononuclear cells from the HIV-1-seronegative donor. Six Gag peptide-specific CD4$^+$ T-cell clones were isolated and their epitopes were mapped. The region of p24 between amino acids 201 and 300 of Gag was defined as the immunodominant region of Gag. A new T helper epitope in the p6 protein of Gag was identified. Two clones were shown to recognize Gag peptides and processed Gag protein, while the other four clones reacted only to Gag peptides under the experimental conditions used. Functional analysis of the clones indicated that both Th1 and Th2 types of CD4$^+$ T cells were obtained. One clone showed direct antigen-specific cytotoxic activity. These clones represent a valuable tool for understanding the cellular immune response to HIV-1, and the study provides new insights into the HIV-1-specific CD4$^+$ T-cell response and the induction of an anti-Gag and -Gag peptide cellular primary immune response in vitro.

Substantial evidence argues for the importance of CD4$^+$ T helper cells in the immune response to human immunodeficiency virus type 1 (HIV-1) infection (47, 55, 56). As in murine models of chronic viral infections, CD4$^+$ T cells may be required for the maintenance of functional HIV-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) responses (38, 46, 56, 70). The lack of CD4$^+$ T-cell responses observed in most HIV-infected individuals could then explain the decline of CTL responses seen over time and the progression of the disease (8, 24). This hypothesis has been strengthened by recent studies that have shown that in long-term nonprogressors and in patients treated with highly active antiretroviral therapy early during primary infection (47, 50, 51, 54, 55), enhanced CD4$^+$ T helper cell responses to HIV-1 are associated with higher levels of HIV-1-specific CTL and with lower viral loads.

Helper T cells could also play a role in viral replication during HIV-1 infection via the regulation and control of the B-cell response and the modulation of neutralizing antibody expression (10, 48), although our data with the hu-PBL-SCID mouse model suggest that neutralizing antibodies are unlikely to be involved in controlling viral replication in established HIV-1 infection (49). Finally, a direct role for CD4$^+$ T cells in the control of viral replication may be possible, via secretion of cytokines and chemokines or cytotoxic activity (45).

The role of CD4$^+$ T cells in the prevention of HIV-1 infection is unclear but likely to be beneficial. Studies have shown that vaccines that stimulate a strong cellular response are largely unable to provide complete protection (18, 59, 69) but can lower viral load and slow disease progression, suggesting that CD8$^+$ and CD4$^+$ T cells can have an impact on infection but cannot provide sterilizing immunity. In contrast, it has been demonstrated that passively administered antibodies can fully protect when high plasma concentrations, unlikely to be achieved through vaccination, are attained (5, 15, 36, 37, 60). Lower levels of antibody have been shown to offer benefit in terms of a delayed and decreased viremia, and it may be that a combination of cellular and antibody responses induced by a vaccine could provide protection. Certainly it seems that an optimal HIV vaccine will elicit strong CTL and antibody responses that will benefit from an efficient CD4$^+$ T-cell response. Some support for this view comes from experiments in the Friend virus model that showed that only a combination of specific CD4$^+$, CD8$^+$, and B cells is able to transfer protection against Friend virus, and any combination of two cell types is insufficient (13).

Understanding and characterizing the HIV-specific CD4$^+$ T-cell response is therefore important for the design of immunotherapies and vaccines for HIV-1 infection. We have previously investigated the impact of CTL and antibodies on established HIV-1 infection with the hu-PBL-SCID mouse model (39, 49). To be able to include CD4$^+$ T cells in these studies, we have begun to generate and characterize HIV-1-specific CD4$^+$ clones. These clones should allow, in future experiments

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in vitro and in the mouse model, the analysis of the interplay of $CD4^+$ T cells, $CD8^+$ T cells, and antibodies in HIV-1 infection.

Very few reports to date have described the characterization of HIV-1-specific $CD4^+$ T-cell clones (11, 28, 44, 52, 68). The cloning of $CD4^+$ T cells is made difficult by the scarcity of these cells in HIV-1-infected persons and by their typical unresponsiveness to HIV-1 antigens (46). Previous studies have suggested that a cellular immune response to HIV-1 proteins can be obtained following in vitro stimulation of cells from HIV-1-negative individuals (6, 29, 31, 32, 40, 61, 65). Furthermore, it has been reported for proteins from other pathogens that T helper epitopes identified following in vitro immunization correspond to epitopes that are immunogenic in vivo (23, 26). This indicates that this type of study can also provide valuable information about the determinants of HIV-1 proteins able to induce primary $CD4^+$ T-cell immune responses in vivo. Therefore we decided to induce an HIV-1-specific $CD4^+$ T-cell response in vitro in peripheral blood mononuclear cells (PBMC) from a normal donor in order to isolate HIV-1-specific $CD4^+$ T-cell clones. We chose the HIV-1 Gag protein as an antigen because a number of recent studies have highlighted the importance of the $CD4^+$ T-cell response to Gag in HIV-1-infected individuals (47, 50, 51, 55).

Here we report the in vitro induction in human PBMC from a seronegative donor of a primary immune response to a mixture of partially overlapping peptides covering the sequence of the HIV-1 IIIB Gag protein. Individual clones with strong reactivity against the Gag peptides were isolated and studied. For each clone, we characterized the peptides recognized, reactivity to processed Gag protein, sensitivity to activation, T-cell receptor (TCR) Vβ usage, major histocompatibility complex (MHC) restriction, and the profile of cytokines secreted. We also determined if cytotoxic activity was present. These results provide new insights into several aspects of the HIV-1 Gag-specific $CD4^+$ T-cell response and the induction of an anti-Gag and -Gag peptide cellular primary immune response in vitro.

MATERIALS AND METHODS

Peptides and reagents. The HIV-1 Gag p55 overlapping 20-mer peptides were obtained from the AIDS Research and Reference Reagent Program (AIDS RRRP), Division of AIDS, National Institute for Allergy and Infectious Disease, National Institutes of Health. The peptides were given an arbitrary number from 1 to 49 following the position in the Gag sequence; full sequences are reported in the AIDS RRRP catalog. The lyophilized peptides were resuspended as indicated by the manufacturers at 1 mg/ml and then stored at −80°C. Two peptides, peptides 35 (P341-360) and 36 (P350-370), were not included in this study because they were not available. To prepare the peptide mixture, the 47 peptides were mixed in equal parts to a final concentration of 1 mg/ml (21 μg/ml for each individual peptide).

Tetanus toxoid (TT) was purchased from Connaught Laboratories Limited, Willowdale, Canada. Anti-CD3 antibody was purchased from Zymed Laboratories, San Francisco, Calif. Human interleukin-2 (IL-2) was obtained from the AIDS RRRP. The recombinant p24 protein was from Austral Biologicals, San Ramon, Calif.

Study subjects and cells. The cells used for the generation of Gag peptide-specific $CD4^+$ T-cell clones were derived from the healthy brother of a pair of monozygotic twins discordant for HIV-1 infection. PBMC were obtained following leukopheresis of the uninfected twin and purification on a Ficoll-Hypaque gradient (Sigma, St. Louis, Mo.). The $CD8^+$ CTL clone specific for HIV-1 Gag (P11-30) was generated from the infected twin as described previously (53) and was a generous gift of S. R. Riddell and P. D. Greenberg. The twins' HLA class II type was DR4, DR7, DQ2, and DQ3, as previously determined by Riddell and colleagues by low-resolution DNA and sequence-specific oligonucleotide probe HLA typing methods (S. Riddell, personal communication). The B lymphoblastoid cell line (B-LCL) was obtained by Epstein-Barr virus transformation of the PBMC from the seronegative twin. As feeder cells for the T-cell cloning, PBMC from healthy volunteers were used after purification by Ficoll-Hypaque density gradient and 4,000-rad γ-irradiation. Class II-negative murine L-cell fibroblasts stably transfected with HLA DR1, DR7, DR4W10, DR4W14, or DR4W15 were obtained from R. W. Karr (25).

Generation of antigen-specific T-cell lines. Gag peptide-specific T-cell lines were generated by culturing PBMC from the uninfected twin with 40 μg of the HIV-1 IIIB Gag peptide mixture (the final concentration of individual peptide was 0.85 μg/ml) per ml in a Costar 24-well plate (Corning, Garden Grove, Calif.) at 3.5×10^6 cells/well. The complete cell culture medium consisted of RPMI 1640 (BioWhittaker, Walkersville, Md.) supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml (all from Life Technologies, Rockville, Md.), and 10% heat-inactivated AB human serum (R10-HS). Cells were incubated at 37°C and 5% CO_2. The control cultures were carried out under the same conditions but with TT at 5 μg/ml as a positive control antigen or without antigen. After 4 days of incubation, the medium was removed and replaced with R10-HS containing 40 U of IL-2 per ml. The cells were grown for an additional 8 to 12 days, with fresh R10-HS/IL-2 medium added every 2 to 3 days. At the end of the in vitro stimulation, the cells were tested for peptide-specific activity.

Generation of T-cell clones. The T cells from the Gag peptide-specific line were cloned by limiting-dilution. The cells were plated at 1, 5, 10, or 25 cells/well in 96-well U-bottomed plates (Corning, Garden Grove, Calif.), and 10^5 heterologous γ-irradiated PBMC were added per well as feeder cells in the presence of anti-CD3 antibody (50 ng/ml) and IL-2 (40 U/ml) in complete medium containing 10% fetal calf serum (FCS) (R10). Four days later the medium was removed and replaced with R10/IL-2 medium. The plates were left untouched for an additional 12 to 14 days. The percentage of wells in which cell growth was detectable was 0, 13, and 26% for plates seeded with 1, 5, and 25 cells/well, respectively.

In order to ensure a high probability of monoclonality, cells were expanded only from the plates that had the lowest percentage of wells in which cell growth was detectable. The clones were transferred into 24-well plates and reactivated in the presence of 2×10^6 irradiated heterologous PBMC as described above. The clones were expanded and then analyzed by fluorescence-activated cell sorting (FACS), screened for antigen-specific proliferative activity, and cryopreserved in aliquots. When needed, the cells were thawed and restimulated as described before and used after 2 to 3 weeks of stimulation.

Proliferation assay. The cells to be analyzed were washed twice in order to remove all IL-2-containing medium and then resuspended in R10-HS as indicated. Cells were seeded in 96-well U-bottomed plates at 10^5 cells/well and incubated with an equal amount of antigen-presenting cells (APC) in a total volume of 200 μl. As APC we used autologous PBMC that were incubated with or without antigen for 2 h and then γ-irradiated (4,000 rad). The antigen was left with the APC in order to give a final concentration in the well of 40 μg/ml for the peptide mixture, 5 μg/ml for the TT, 2.5 μg/ml for the recombinant p24, 0.5 μg/ml for the single peptides, or otherwise as indicated. Each condition was assayed in duplicate.

In some experiments, proliferation was also determined following nonspecific activation with 50 ng of anti-CD3 antibody and 40 U of IL-2 per ml. Three days later, the cells were pulsed with [^{3}H]thymidine (ICN Biomedicals, Irvine, Calif.) at 1 μCi/well, and uptake was measured in a β-scintillation counter after 18 h. The incubation time was extended to 5 days before the addition of [^{3}H]thymidine for assays involving freshly isolated PMBC. In some experiments, the results are reported as stimulation index. The stimulation index is the ratio of the level of proliferation, in counts per minute, obtained in the presence of antigen and the level of proliferation obtained without antigen.

TCR Vβ analysis. Following lysis of T-cell clones and PBMC in Trizol solution (Gibco-BRL, Carlsbad, Calif.), total RNA was prepared by chloroform extraction and isopropanol precipitation. The RNA was reverse transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Carlsbad, Calif.) and poly(T) primers (Roche, Basel, Switzerland). Analysis of TCR Vβ usage was performed with the TCR typing amplimer kit (Clontech, Palo Alto, Calif.) according to the manufacturer's instructions. The cDNA from each clone was tested with a panel of 25 sense primers, each specific for one Vβ sequence, and an antisense primer located in the Cβ region. Primers specific for the TCR α chain constant region (Cα) were used as a positive control. The PCR products were analyzed on a 1.8% agarose gel.

For clones 6 and 74, analysis of the TCR Vβ usage was further carried out by cloning and sequencing the complete variable region of the TCR β chain. The

TCR Vβ region was reverse transcribed and PCR amplified with the Smart Race cDNA amplification kit from Clontech as specified by the manufacturer's instructions and the Cβ antisense primer provided with the Amplimer kit (Clontech). PCR products were cloned with the Topo TA cloning kit (Invitrogen, Carlsbad, Calif.). Double-stranded plasmid DNA was isolated from 13 and 6 colonies for clones 6 and 74, respectively. Sequences were obtained by automated sequencing (ABI, Perkin-Elmer, Foster City, Calif.) with the M13 reverse primer.

Two primers specific for the Vβ6s5A1N1 and Vβ17Vs1A1T sequences (5′-AATGAAGCTCAACTAGAA-3′ and 5′-CAGATAGTAAATGACTTT-3′, respectively) were designed and used for PCR amplification with the cDNA of clone 6 as a template.

Flow cytometry analysis and intracellular cytokine staining. Cells were stained with fluorochrome-conjugated antibodies to CD4 (clone S3.5) and to CD8 (clone 3B5) from Caltag Laboratories (Burlingame, Calif.) in phosphate-buffered saline (PBS) containing 1% FCS and 0.02% NaN_3 (Sigma, St. Louis, Mo.). Stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Diego, Calif.) with CellQuest software (Becton Dickinson).

For intracellular cytokine staining, cells at days 14 to 18 of activation were plated in a 96-well flat-bottomed plate at 10^6 cells/well in 200 μl of R10/IL-2 and incubated at 37°C in the presence of different antigens or without antigen. Single peptides were used at 0.5 μg/ml, anti-CD3 antibody at 50 ng/ml, and vaccinia virus-infected cells at 0.5×10^6 cells/well. GolgiPlug (Pharmingen, San Diego, Calif.), a reagent that contains brefeldin A, was added to the cultures to inhibit protein transport and allow the detection of the cytokines. In some cases, the antibodies anti-HLA DR L243 (clone G46-6) from Pharmingen or anti-HLA DQ (clone SPVL3) from Coulter (Coulter, Westbrook, Maine) were added to the cultures at 0.5 μg/ml. After 6 h, cell surface staining was performed as described above, followed by intracellular cytokine staining with a Cytofix/Cytoperm kit (Pharmingen, San Diego, Calif.) in accordance with the manufacturer's recommendations. The following antibodies were used: anti-gamma interferon (IFN-γ)-phycoerythrin (PE) conjugate (clone 4S.B3), anti-IL-2–allophycocyanin (clone MQ1-17H12), anti-IL-4–fluorescein isothiocyanate (FITC) conjugate (clone MP4-25D2), and anti-IL-5–PE conjugate (clone JES1-39D10) (Pharmingen, San Diego, Calif.).

To characterize the HLA DR subtype that presents peptide 271-290 to clone 6, intracellular cytokine staining was performed following activation of clone 6 by HLA DR-transfected fibroblasts pulsed with the specific peptide. HLA DR1-, DR7-, DR4W10-, DR4W14-, and DR4W15-transfected fibroblasts were plated in a 24-well plate at 10^6 cells/well and incubated for 2 h with peptide 271-290 at a concentration of 4 μg/ml. The cells were then washed seven times with R10 to remove all unbound peptide, and 10^6 clone 6 T cells were added per well in the presence of brefeldin A. After 6 h, the cells in suspension were analyzed as described above.

Vaccinia virus infection. The following reagents were obtained through the AIDS RRRP: vP1170, wild-type vaccinia virus, contributed by Daniel R. Kuritzkes; and vDK1, recombinant vaccinia virus containing the coding region for HIV-1 Gag, contributed by Virogenetics Corp. Vaccinia virus stocks were diluted 1:2 in trypsin-EDTA solution for cell culture (Life Technologies, Rockville, Md.) and incubated for 30 min at 37°C, vortexing every 10 min. The virus was added to B-LCL at a multiplicity of infection (MOI) of 0.3 in a total volume of 2.5 ml of R10 in a six-well plate, and the cells were incubated for 18 h at 37°C. Vaccinia virus-infected cells were used as targets in a cytotoxicity assay and to evaluate T-cell activation induced by Gag-expressing cells.

Cytotoxicity assay. Vaccinia virus-infected and peptide-pulsed autologous B-LCL were used as target cells in a standard chromium release assay. Target cells were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ (Amersham Pharmacia Biotech, Piscataway, N.J.) for 2 h at 37°C and washed three times with R10. The cells were then seeded in U-bottomed 96-well microtiter plates at 2×10^4 cells/well. For peptide-pulsed target cells, pulsing was performed overnight with the peptide mixture at 10 μg/ml, and the peptides were added again at a final concentration of 2.5 μg/ml following chromium labeling.

Effector cells were added, in triplicate, in a final volume of 200 μl. Single T-cell clones at days 15 to 18 of activation were used as effector cells at an effector-to-target cell (E:T) ratio ranging from 0.5 to 20. The plates were incubated at 37°C for 6 h. The supernatants were then harvested (100 μl), and radioactivity was counted in a gamma-counter. Maximal release of radioactivity was determined by lysis of target cells in 1% Empigen BB (Calbiochem, San Diego, Calif.). Spontaneous release was measured in the absence of effector cells. The percent specific lysis was determined from the formula [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release was less than 30%.

RESULTS

Isolation of Gag peptide-specific limiting-dilution CD4$^+$ T-cell clones from a seronegative donor. The in vitro isolation of antigen-specific T cells from nonimmune individuals is difficult because the frequency of antigen-specific cells is low. However, a few reports have shown that it is sometimes possible to induce in vitro an immune response to certain antigens and to isolate the responsive T cells (23, 26, 30, 31, 61). Here we report the induction of a CD4$^+$ T-cell response to HIV-1 Gag peptides in PBMC from an HIV-1-seronegative individual and the subsequent isolation of Gag- and Gag peptide-specific CD4$^+$ T-cell clones from the stimulated PBMC.

PBMC of an HIV-1-seronegative donor were stimulated in vitro with a mixture of 47 partially overlapping 20-mer peptides covering the whole sequence of the HIV-1 Gag protein, with the exception of 10 amino acids at the C-terminal end of p24. Four days later, the peptides were removed, and IL-2-containing medium was added. The cells were then grown for 2 weeks and tested for the presence of a specific proliferative response. Irradiated autologous PBMC were used as APC and added to the in vitro-stimulated T cells in the presence of the HIV-1 Gag peptide mixture or of TT. After a 3-day incubation with the antigen, incorporation of [^{3}H]thymidine was measured. Based on previous experiments of in vitro immunization, a proliferative response higher than twice the proliferative response obtained in the absence of antigen was considered significant.

As shown in Fig. 1A, the cells did not proliferate in the absence of antigen or in response to TT but proliferated significantly in response to the HIV-1 Gag peptide mixture. The magnitude of peptide-specific proliferation increased when the concentration of the peptide mixture was doubled from 3 to 6 μg/ml but decreased slightly if the concentration was further increased to 12 μg/ml. As shown in Fig. 1B, the same proliferation assay performed on the PBMC before the in vitro stimulation did not reveal any measurable proliferation in response to the HIV-1 Gag peptide mixture. However, the cells did proliferate in response to the recall antigen TT. This result indicates that the in vitro immune activation performed on the PBMC successfully triggered the expansion of a population of previously undetectable HIV-1 Gag peptide-specific T cells.

In order to isolate individual specific T-cell clones, the Gag peptide-specific line was stimulated nonspecifically with an anti-CD3 antibody and plated in limiting-dilutions. Limiting-dilution T-cell clones were isolated so as to ensure a high probability of monoclonality, as explained in Materials and Methods, and their CD4$^+$ or CD8$^+$ phenotype was analyzed by FACS. Sixteen limiting-dilution clones were determined to be CD4$^+$ CD8$^-$ T cells and 16 were CD8$^+$ CD4$^-$ T cells. The CD4$^+$ T-cell clones were individually tested in a proliferation assay for Gag peptide-specific reactivity as described above (data not shown). Of the 16 CD4$^+$ T-cell clones tested, 6 showed an HIV-1 Gag peptide-specific proliferation and were expanded for further characterization.

We also investigated whether any of the limiting-dilution CD8$^+$ T-cell clones were specific for the HIV-1 Gag protein or peptides. A standard ^{51}Cr release assay was performed, as described below, to determine the existence of Gag-specific lysis of target cells. None of the CD8$^+$ T-cell clones showed

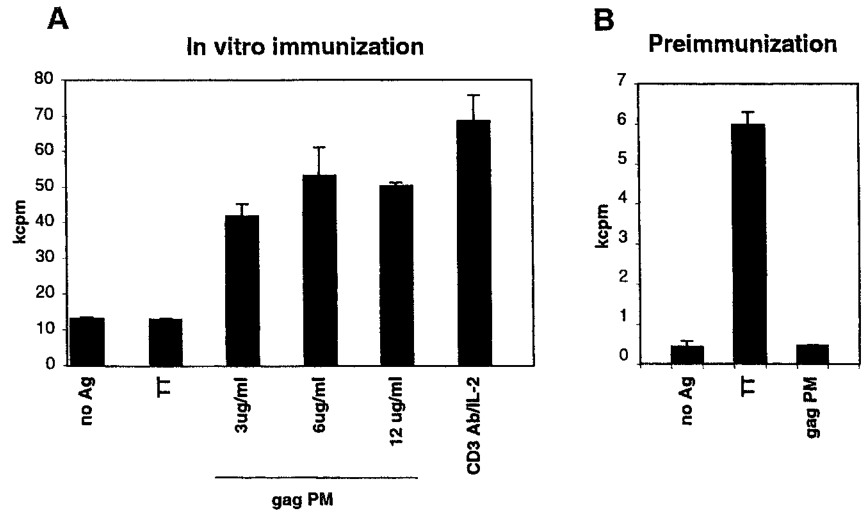

FIG. 1. Proliferative response of cells from an HIV-1-seronegative individual before and after in vitro immunization. The T-cell line obtained after 2 weeks of in vitro stimulation with HIV-1 Gag peptides (A) or preimmunization PBMC (B) were analyzed for antigen-specific proliferation. The cells were stimulated with (i) 5 μg of TT per ml, (ii) Gag peptide mixture (PM) as indicated, (iii) 50 ng of anti-CD3 antibody (Ab) and 50 U of IL-2 per ml, or (iv) no antigen (Ag). Irradiated autologous PBMC were added as APC to the T-cell line (B). [^{3}H]thymidine uptake was measured after 3 days (A) or 5 days (B) and is reported as total 10^3 counts per minute (kcpm).

any detectable Gag- or Gag peptide-specific cytotoxic activity (data not shown).

Identification of peptides recognized by Gag peptide-specific limiting-dilution CD4$^+$ T-cell clones. In order to identify the peptides specifically recognized by the 6 Gag peptide-specific limiting-dilution CD4$^+$ T-cell clones, proliferation assays were performed in which each clone was tested against each of the 47 individual peptides of the Gag peptide mixture used for the original in vitro stimulation. The results are reported in Fig. 2 and Table 1. Each clone showed a specific pattern of stimulation. All the epitopes identified were peptides from the p24 region of the HIV-1 Gag protein, with the exception of one peptide, recognized by clone 74, corresponding to the C terminus of the p6 protein. In the p24 protein the epitopes of the Gag peptide-specific CD4$^+$ T-cell clones were mostly located between HIV-1 Gag residues 201 and 300 (Fig. 2). Moreover, peptide 271-290 was recognized by three of the six clones. As discussed below, TCR analysis excluded that these three clones were identical. This result was also confirmed by the different patterns of cytokine secretion of these clones upon antigen stimulation, as described below. Interestingly, clones 6 and 74 recognized three and two different peptides of HIV-1 Gag, respectively. Therefore, to understand whether these limiting-dilution clones were truly clonal or were composed of mixed cell populations, we analyzed their TCR Vβ region usage.

Characterization of TCR of Gag peptide-specific limiting-dilution CD4$^+$ T-cell clones. To analyze the TCR Vβ gene expression of the limiting-dilution CD4$^+$ T-cell clones, cDNA was prepared from each clone and used as a template for PCR amplification with a panel of 25 Vβ family-specific primers. A representative example is given in Fig. 3A. As a positive control for the primer panel, we used cDNA prepared from PBMC (Fig. 3A). For clones 37 and 50, the amplification gave a product with only one of the primers used, corresponding to the Vβ3 and Vβ17 families, respectively, confirming that these clones were composed of a homogenous T-cell population with a single Vβ usage. This result strongly suggested that the cells were clonal, especially as they were shown to react to a single peptide. For clone 74, the PCR amplified a product with only one primer, corresponding to the Vβ13 family. However, as clone 74 has been shown to react to two different peptides, the possibility still existed that the cell population was composed of two clones with the same Vβ region but with different VDJ rearrangements. Therefore, analysis of the TCR of T-cell clone 74 was carried out further, as described below. For clones 85 and 97, amplification products were obtained with two of the tested primers, indicating mixed cell populations. Surprisingly, PCR with the cDNA of clone 6 did not give any amplification product, suggesting the usage of a Vβ family not represented in the panel of primers that we used.

Analysis of the TCR of clones 74 and 6 was completed by cloning and sequencing of the TCR β-chain gene. Sequencing of 13 colonies from clone 6 resulted in 11 identical sequences, corresponding to the s5A1N1 subtype of the Vβ6 family, and in two Vβ17s1A1T sequences (4). As no product was previously amplified with the Vβ17-specific primer, it was likely that the two latter sequences represented a DNA contamination from the irradiated feeder cells used to grow the T-cell clones. In order to verify this hypothesis, we designed two primers specific for the Vβ6s5A1N1 and Vβ17Vs1A1T sequences and used them for amplification with the cDNA of clone 6 as a template. Only the primer specific for Vβ6s5A1N1 gave a product with the clone 6 cDNA (Fig. 3B), while both primers gave a product with control cDNA (data not shown), strongly suggesting that clone 6 was indeed a clonal population of cells.

For clone 74, the sequencing of six clones following the cloning of the Vβ gene revealed an identical Vβ13s1 sequence and an identical VDJ rearrangement. Furthermore, cloning and sequencing of the product obtained after amplification with the Vβ13-specific primer also showed identical sequences

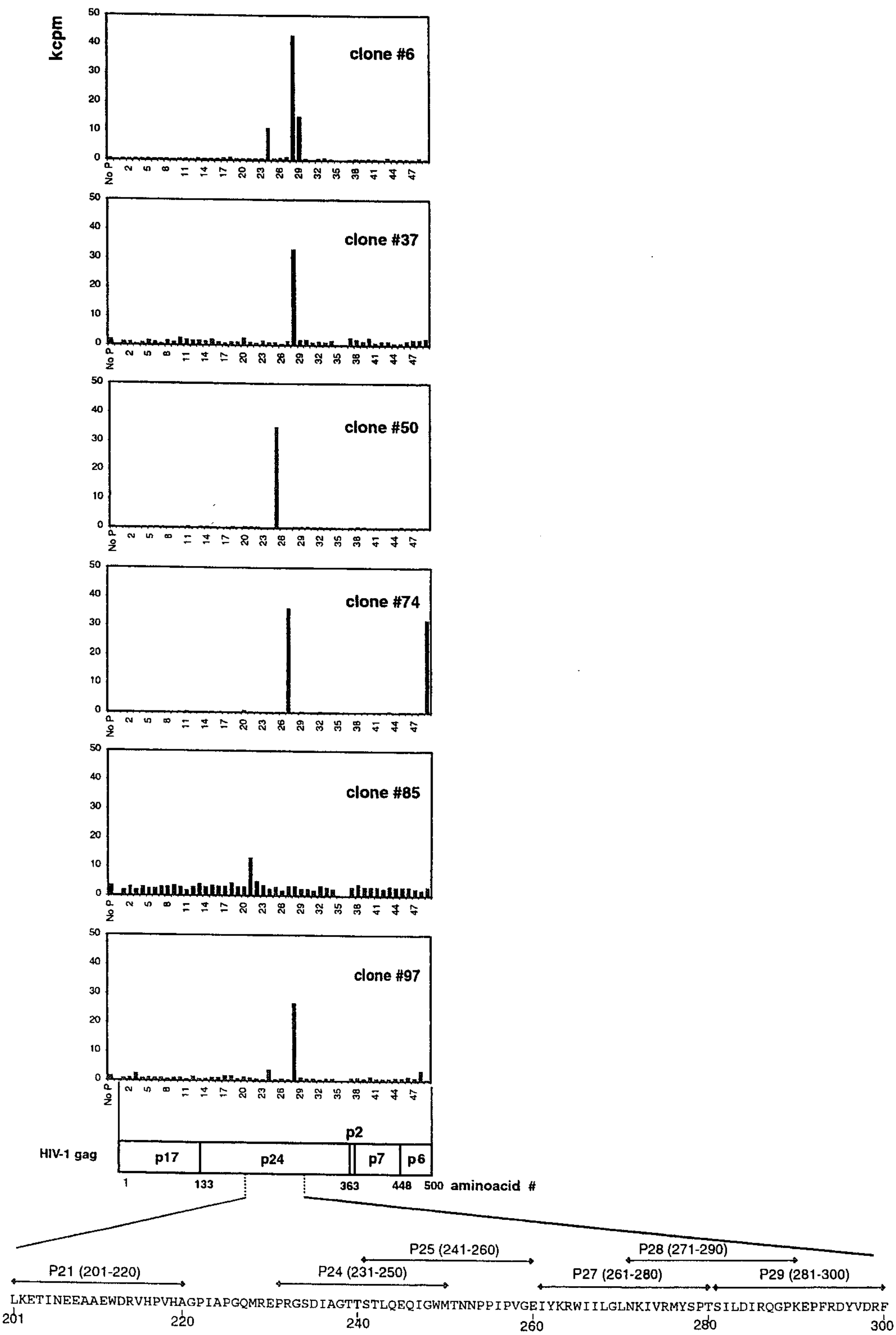

FIG. 2. Identification of peptides recognized by CD4+ T-cell clones. T cells from the indicated clone were incubated with each of the 49 peptides of HIV-1 Gag protein (except for the peptides 35 and 36) at 0.5 μg/ml in the presence of irradiated autologous PBMC. Tritiated thymidine uptake was measured at day 3 and is reported as total 10^3 counts per minute. In the figure, No P indicates the proliferation level in the absence of peptide. A representation of the HIV-1 Gag protein is shown to illustrate the position of each peptide along the sequence of Gag. The central region of p24 has been enlarged to show the positions of some of the peptides recognized by the T-cell clones.

TABLE 1. Characteristics of HIV-1-specific CD4$^+$ T-cell clones

Clone no.	Peptide			Th subtype	TCR Vβ
	No.	Positions in Gag	Sequence		
6	29	281–300	SILDIRQGPKEPFRDYVDRF	Th1	6 (6s5A1N1)
	28	271–290	NKIVRMYSPTSILDIRQGPK		
	24	231–250	PRGSDIAGTTSTLQEQIGWM		
37	28	271–290	NKIVRMYSPTSILDIRQGPK	Th2	3
50	25	241–260	STLQEQIGWMTNNPPIPVGE	Th0	17
74	27	261–280	IYKRWIILGLNKIVRMYSPT	Th1	13 (13s1)
	49	480–500	DKELYPLTSLRSLFGNDPSSQ		
85	21	201–220	LKETINEEAAEWDRVHPVHA	Mixed	8, 18
97	28	271–290	NKIVRMYSPTSILDIRQGPK	Mixed	9, 14

in four clones, further confirming the true clonality of limiting-dilution T-cell clone 74. In conclusion, our results suggest that limiting-dilution T-cell clones 6, 37, 50, and 74 are true clonal cell populations, while 85 and 97 consist of mixed cell populations. Nevertheless, since lines 85 and 97 exhibit functional clonal behavior, we shall henceforth refer to these two lines and the four true clones collectively as clones, according to the convention adopted by Norris et al. (44).

Determination of sensitivity to peptide activation of Gag peptide-specific T-cell clones. In order to understand whether the Gag peptide-specific CD4$^+$ clones isolated from the HIV-1-negative individual reacted with physiological concentration of their specific peptides, we performed proliferation assays with various concentrations of peptides. Cells were incubated with concentrations of peptide from 0.03 ng/ml to 2 μg/ml, and proliferation was measured as described above. The results are shown in Fig. 4. Most clones started to proliferate in response to concentrations of peptide ranging from 5 to 50 ng/ml (2.5 to 25 nM) (Fig. 4A). Interestingly clone 6 showed an extremely high sensitivity to stimulation with peptide 271-290, starting to proliferate in response to concentrations of this peptide as low as 0.3 ng/ml (0.15 nM). Clone 6 also proliferated specifically in response to peptides 231-250 and 281-300, as expected from the epitope mapping experiments, but at higher concentrations, suggesting that peptide 271-290 contains the main epitope of this clone (Fig. 4B). Clone 74 was stimulated by two distinct peptides but at very different concentrations, 200 ng/ml (100 nM) and 1 μg/ml (0.5 μM) for peptides 480-500 and 261-280, respectively (Fig. 4C). The cell line 85 demonstrated a specific reactivity to peptide 201-220 but only at high concentrations, and even at such concentrations, the proliferation level remained low.

Analysis of cytokines secreted by Gag peptide-specific CD4$^+$ T-cell clones upon stimulation. Two subtypes of CD4$^+$ T helper cells, T helper 1 (Th1) and T helper 2 (Th2), have been described, differing in functional role and pattern of cytokine secretion. Typically, Th1 cells secrete IL-2 and IFN-γ, providing support for cellular immune responses, although IL-2 secretion in human T cells is not as restricted to the Th1 subset as in mouse T cells (43). Th2 cells secrete IL-4 and IL-5 among

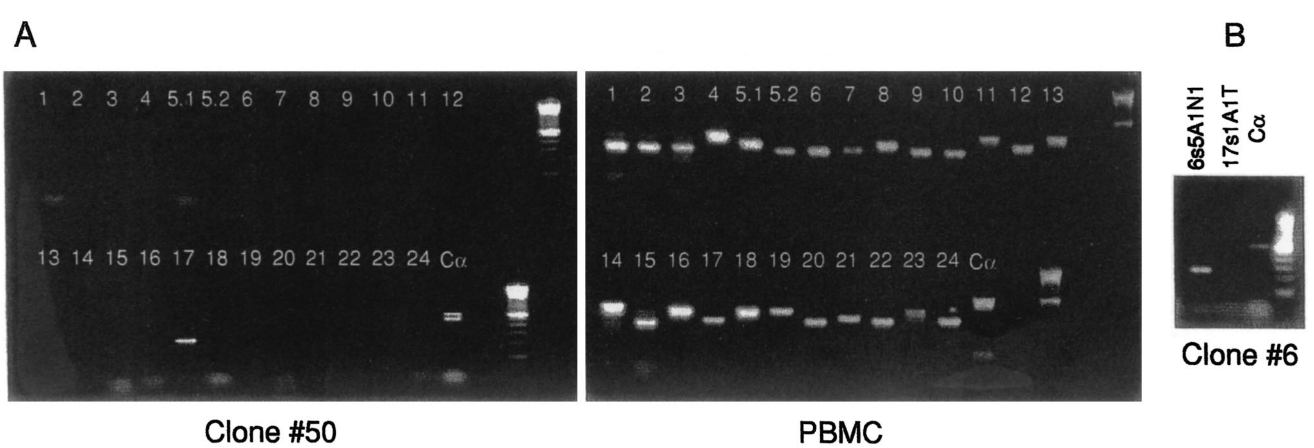

FIG. 3. TCR characterization of T-cell clones by reverse transcription-PCR. (A) A set of 25 primers, specific for each Vβ gene family, was used to perform reverse transcription-PCR in order to determine the Vβ usage of each T-cell clone. Clone 50 is shown here as a representative example. cDNA from PBMC was used as a control for the primer set. (B) Reverse transcription-PCR on clone 6 with primers specific for two subvariants of Vβ 6 and Vβ 17 (6s5A1N1 and 17s1A1T, respectively) identified by sequencing of the TCR of clone 6. A pair of primers annealing to the constant region of TCR α chain were included as a positive control (Cα).

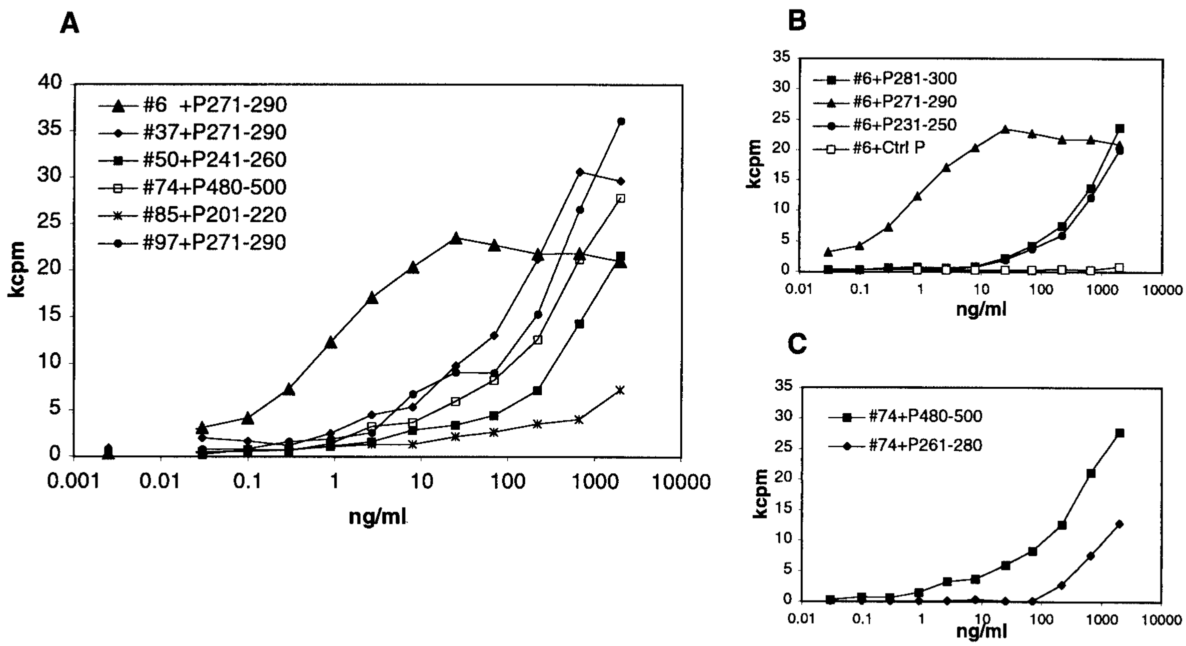

FIG. 4. Sensitivity of HIV-1 Gag peptide-specific CD4$^+$ T-cell clones to activation mediated by individual peptides. Each CD4$^+$ T-cell clone was stimulated with different concentrations of the indicated peptide in the presence of irradiated autologous PBMC. Ctrl P indicates proliferation in the presence of an irrelevant peptide. The lymphoproliferative response was measured after 3 days by [^{3}H]thymidine uptake and is expressed as total 10^3 counts per minute.

other cytokines and preferentially support the humoral immune response. In order to determine the Th1 or Th2 phenotype of the six HIV-1 Gag peptide-specific CD4$^+$ T-cell clones, we determined the cytokines produced following stimulation by intracellular staining and FACS analysis. The cells were first stimulated with anti-CD3 antibody or with their specific peptide in the presence of brefeldin A, a compound that blocks extracellular secretion. After 6 h the cells were fixed and permeabilized to allow intracellular detection of cytokines by antibodies conjugated to fluorochromes. We determined the cytokine production profile of each of the HIV-1 Gag peptide-specific CD4$^+$ clones in response to activation. Figure 5 shows the intracellular production of IFN-γ and IL-2 (panel A) and IL-4 and IL-5 (panel B) for three representative clones in response to anti-CD3 antibody or peptide activation. The T helper phenotypes of the clones are summarized in Table 1.

All the CD4$^+$ clones expressed high levels of IL-2 when stimulated with their specific peptides, and therefore we based the distinction between Th1 and Th2 on the detection of IFN-γ, IL-4, and IL-5. As described by others, IL-4 staining is weak for stimulated CD4$^+$ T cells, but it is still sufficient to allow Th1-Th2 discrimination. The two clones 6 and 74 could be clearly defined as Th1 because they expressed high levels of IFN-γ and did not express IL-4 and IL-5. Of note, following activation of clone 6 with its specific peptide, 100% of the cells responded by cytokine production, confirming the clonal nature of the cell population. Clone 37 was characterized as Th2 because it did not express IFN-γ and expressed IL-4. Clone 50 expressed IFN-γ, IL-4, and low levels of IL-5 and was thus defined as Th0, a subset of T cells characterized by the secretion of both Th1 and Th2 types of cytokines (42, 63). Triple staining confirmed that the same cells were able to produce IL-2, IFN-γ, and IL-4 (data not shown). The remaining two cell lines, 85 and 97, were confirmed to be composed of a nonclonal population of cells, as discussed previously, because the IFN-γ-positive cells and the IL-4-positive cells constituted two distinct populations. The cytokine production of these two lines was low upon peptide stimulation, suggesting the presence of a relatively small percentage of peptide-specific cells. An alternative explanation is that intracellular cytokine staining was not as sensitive as the proliferation assay in reporting on T-cell activation, at least in these instances.

HLA class II restriction of Gag peptide-specific CD4$^+$ T-cell clones. Intracellular cytokine FACS staining was also used to identify the HLA class II restriction of peptide recognition by the Gag peptide-specific CD4$^+$ T-cell clones. The donor used for the cloning was DR4, DR7, DQ2, and DQ3. Each clone was incubated with the corresponding peptide with or without the addition of anti-DR or anti-DQ antibody, and the assay was performed as described before. The peptide-mediated activation of the T-cell clones tested was significantly blocked by the anti-DR antibody and not affected by the anti-DQ antibody, as shown for one representative clone in Fig. 6A. The anti-DR antibody did not inhibit the nonspecific activation of the clones mediated by the anti-CD3 antibody (data not shown). This result suggested that peptide recognition was restricted by HLA class II DR molecules in all the clones.

Since additional experiments with clone 6 were planned, we extended the HLA restriction analysis for this clone. A panel of HLA DR1-, DR7-, DR4W10-, DR4W14-, and DR4W15-transfected fibroblasts were used as antigen-presenting cells, and activation of clone 6 was detected by intracellular IL-2 staining (25). Clone 6 was activated only when its specific peptide was presented by fibroblasts transfected with the HLA

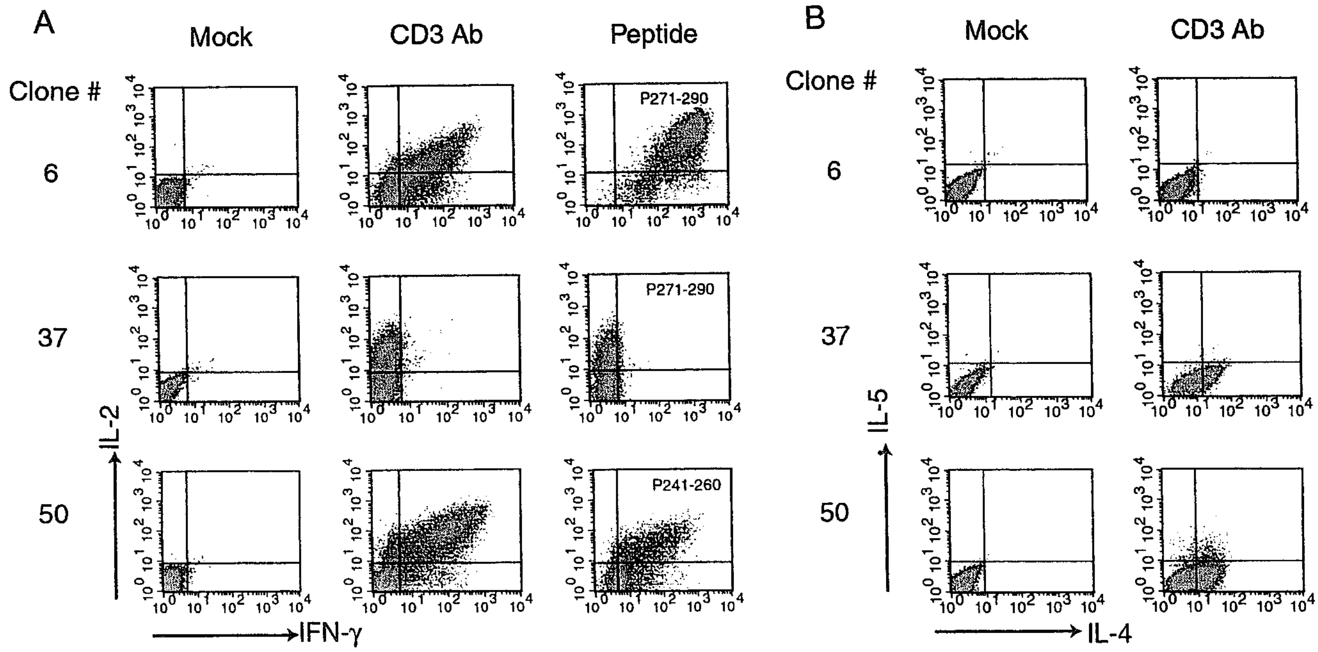

FIG. 5. Intracellular cytokine production by Gag peptide-specific CD4$^+$ T-cell clones. Cells from the indicated CD4$^+$ T-cell clones were incubated with anti-CD3 antibody (50 ng/ml) or with the indicated peptide (0.5 μg/ml) for 6 h in the presence of brefeldin A and then stained intracellularly with fluorescently labeled antibodies for FACS analysis. Cells were double-stained with anti-IFN-γ–PE and anti-IL-2–Apc (A) or with anti-IL-4–FITC and anti-IL-5–PE (B).

DR4 subtype W14 or W15 (Fig. 6B). No stimulation was detected with fibroblasts expressing HLA DR1, DR7, or DR4W10. Of note, upon activation, clone 6 induced a cytopathic effect in the adherent layer of fibroblasts expressing HLA DR4W14 and -W15, leading to detachment from the plate of some cells visible in the FACS analysis as an additional CD4$^-$ IL-2$^-$ population of cells.

Analysis of activation of CD4$^+$ T-cell clones in response to

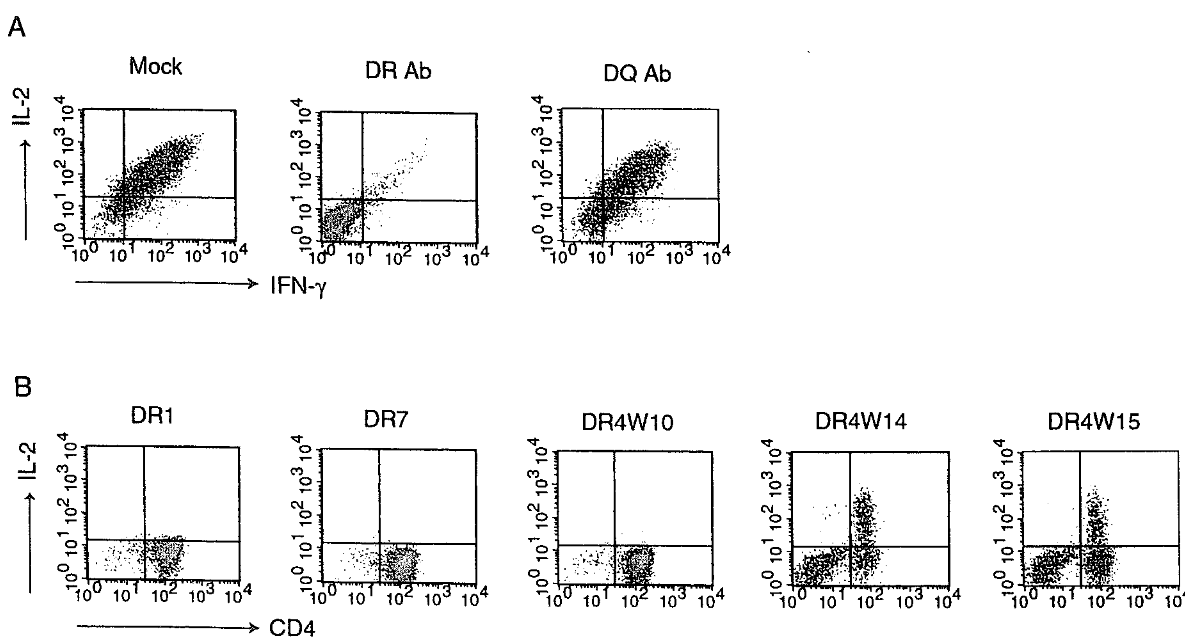

FIG. 6. HLA class II restriction of CD4$^+$ T-cell clone 6 recognition of peptide 271-290. (A) T cells were incubated with 0.5 μg of peptide 271-290 per ml and brefeldin A in the presence of 0.5 μg of anti-DR or anti-DQ antibodies per ml or without antibody (Ab) for 6 h. Intracellular FACS staining was performed with anti-IFN-γ–PE and anti-IL-2–Apc antibodies. (B) Fibroblasts stably transfected with the indicated HLA molecules were seeded on plates and pulsed for 2 h with peptide 271-290. The excess peptide was removed, and the cells of clone 6 were added to the fibroblasts in the presence of brefeldin A. After 6 h, the nonadherent cells were stained extracellularly with anti-CD4–PE and intracellularly with IL-2–Apc.

vaccinia virus-expressed Gag and to recombinant p24. The 20-mer peptides used here do not necessarily correspond to peptides that would be produced following cellular processing of the HIV-1 Gag protein during the course of HIV-1 infection. Antigen processing into small peptides and the presentation of these peptides on HLA class II depends on multiple factors and is hard to predict. To determine whether the peptides recognized by the clones that we have isolated could be presented following HIV-1 infection, we first studied the reactivity of these clones to cells infected with a Gag-expressing vaccinia virus. We chose to use a recombinant vaccinia virus instead of the original HIV-1 in order to obtain a greater number of infected, Gag-expressing cells. An autologous B-LCL was used as an APC and infected overnight with a recombinant vaccinia virus containing the gene for the HIV-1 IIIB Gag protein. The infected B-LCLs were mixed with the $CD4^+$ T-cell clones for 6 h, and intracellular cytokine staining was performed as described above to detect $CD4^+$ T-cell stimulation. Clones 6 and 74 showed signs of activation in response to vaccinia virus Gag-infected B-LCL while showing no sign of activation with B-LCL infected with the wild-type vaccinia virus (Fig. 7A). The other four clones did not display detectable activation in response to vaccinia virus Gag-infected B-LCL.

The six clones were also tested for proliferative activity in response to the p24 protein. In this experiment, autologous PBMC were used as APC and pulsed with recombinant p24. As shown in Fig. 7B, clone 6 showed a strong specific proliferation in response to p24 as well as to the cognate peptide 271-290. The other clones did not show a specific proliferation in response to recombinant p24 (data not shown). Of note, clone 74, which recognizes epitopes from p24 and p6, was activated by vaccinia virus-Gag-infected cells but not by recombinant p24.

In conclusion, the results show that the epitopes contained in the 20-mer peptides specifically recognized by clones 6 and 74 are also contained in peptides presented upon processing of the HIV-1 Gag protein. The failure of the other clones to be activated by recombinant p24 or by vaccinia virus-expressed Gag may indicate that proteolytic degradation of the Gag protein does not lead to generation of the requisite peptides. An alternative explanation is that the peptides are produced upon processing but that the density of MHC molecules endogenously loaded with the requisite peptide is too low to induce detectable activation of the clones. In contrast, incubation with exogenous peptides at a high concentration may result in a high density of occupied MHC molecules and in cell activation (7). This hypothesis may be especially applicable to the clones that demonstrated a lower sensitivity to peptide activation (Fig. 4). In addition, the intracellular cytokine staining and proliferation assay may not be sufficiently sensitive to detect cell activation under suboptimal conditions. Finally, it cannot be totally ruled out that these clones would be activated in vivo, since the processing of Gag by APC in vitro in the systems we used differs from the in vivo processing by professional APC during HIV infection. The fact that the characterized peptides correspond to peptides described in HIV-positive donors argues in favor of the latter hypothesis, as explained in Discussion.

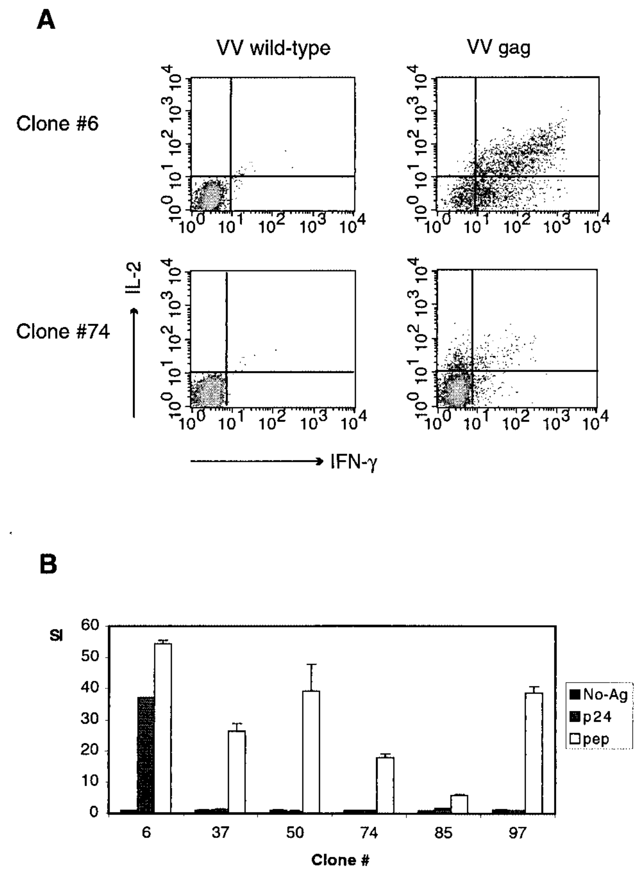

FIG. 7. (A) Activation of $CD4^+$ T-cell clones 6 and 74 by recombinant vaccinia virus-infected cells expressing HIV-1 Gag. B-LCL were infected overnight with wild-type vaccinia virus (VV) or vaccinia virus containing the HIV-1 Gag gene and then added to the T cells for 6 h in the presence of brefeldin A. The cells were stained with anti-CD4–FITC antibody and then used for the intracellular detection of IFN-γ and IL-2. The plots were generated by gating on the CD4-positive cells. (B) Proliferation of the $CD4^+$ T-cell clones in response to recombinant p24. Autologous PBMC were pulsed with p24 (2.5 μg/ml), the cognate peptide (pep) (2.5 μg/ml), or without antigen (No-Ag) and then added to the T cells. Tritiated thymidine uptake was measured at day 3 and is reported as the stimulation index (SI).

Cytotoxic activity. Cytotoxic activity is usually associated with $CD8^+$ CTL but can also be detected in $CD4^+$ T cells, in particular those of the Th1 subset (44, 45). Therefore, we analyzed the Gag peptide-specific $CD4^+$ clones for the presence of cytotoxic activity with a standard ^{51}Cr release assay. The Gag-specific $CD8^+$ CTL used as a positive control displayed strong cytotoxic activity (Fig. 8). Among the $CD4^+$ clones, only clone 6 displayed a moderate but significant Gag-specific cytotoxic activity, with 20% specific lysis at an effector-to-target ratio of 5:1.

DISCUSSION

Several studies have reported that nonimmune PBMC can be primed in vitro and an immune response that was not previously detectable can be subsequently stimulated (23, 26, 30, 31, 61). Here we have successfully induced, in PBMC from

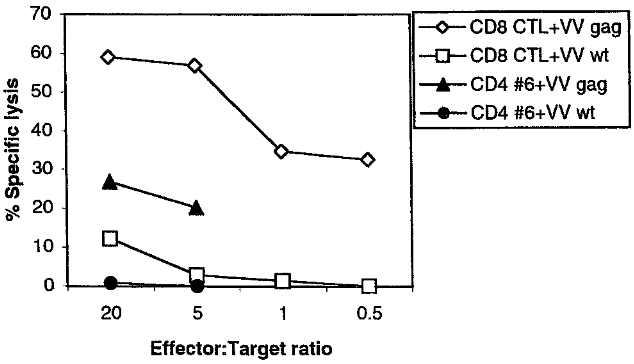

FIG. 8. Cytotoxic activity of HIV-1-specific CD4$^+$ T-cell clone 6 compared to an HIV-1-specific CD8$^+$ cytotoxic T-cell clone (CTL). A standard ^{51}Cr release assay was performed by incubating the T cells as effector cells with ^{51}Cr-labeled B-LCL infected with either wild-type (wt) vaccinia virus (VV) or vaccinia virus expressing HIV-1 Gag as target cells. Different amounts of CD4$^+$ T cells from clone 6 or of CD8$^+$ T cell from the control clone were incubated with equal amounts of ^{51}Cr-labeled vaccinia virus-infected B-LCL, and ^{51}Cr release was measured after 6 h.

an HIV-1-seronegative donor, an immune response to a mixture of peptides representing the whole HIV-1 IIIB Gag protein and generated HIV-1 Gag- and Gag peptide-specific CD4$^+$ T-cell clones.

In our experimental conditions, in contrast to some other reports (6, 29, 32), the use of additional professional APC such as dendritic cells was not required, and PBMC alone were sufficient to stimulate antigen-specific T CD4$^+$ cells in vitro. The PBMC did not initially show Gag peptide-specific responses, as measured by a proliferation assay (Fig. 1) and by intracellular cytokine staining (data not shown), but did respond to the peptides following in vitro immunization. This is consistent with the hypothesis that naive T cells have been primed during the immunization. However, it cannot be ruled out that the CD4$^+$ T-cell response obtained was due to secondary activation of memory T cells specific for cross-reacting antigens, i.e., the HIV-1 Gag peptides stimulated memory T cells originally elicited by a previous encounter with an unknown cross-reactive antigen.

The presence of sequence homology between antigens is one possible mechanism to explain cross-reactivity that could lead to the observed CD4$^+$ T-cell response. Therefore, the sequence of peptide 271-290, which was recognized by three different clones that we characterized, was used to perform a homology search of the GenBank protein database. The only partial homology we found was in a protein of xestia c-nigrum granulovirus, a non-human pathogen, which we did not consider biologically relevant. Another mechanism for antigen cross-reactivity is based on degeneracy of TCR recognition (21, 22); proteins from other pathogens and of unrelated sequences may induce an immune response that cross-reacts with HIV-1.

Investigation of the specificity of the CD4$^+$ T-cell clones isolated upon in vitro immunization revealed seven peptides that induced a specific proliferative response. Two clones, 6 and 74, recognized more than one peptide. We have shown, by characterization of their TCR Vβ chain, that their cell population is truly clonal. Therefore, the ability of these clones to recognize different peptides must be explained by the degeneracy of TCR recognition. Recent studies have demonstrated the great flexibility of the TCR with respect to recognition of peptides bound to self-MHC (21) and suggest that the specificity of T-cell clones can be defined by sets of TCR ligands rather than by single unique structures (62). Moreover, examples of clones recognizing two different epitopes in the same viral protein or in two different proteins of the same virus have been described previously (3, 27).

Most of the CD4$^+$ T-cell clones recognized peptides corresponding to sequences in p24. Only one peptide corresponding to the C terminus of p6 and no peptide corresponding to p2, p7, or p17 elicited a response. The conserved region of p24 between amino acids 201 and 300 of Gag could be defined as the immunodominant region of Gag under our experimental conditions, since six of the identified peptides correspond to sequences in this region (Fig. 2). Among the peptides that we identified, peptide 271-290 seemed to be the immunodominant one, since it was recognized by three of the six clones we isolated, 6, 37, and 97. These clones are clearly distinct, as shown by characterization of TCR Vβ usage. Interestingly, two groups have previously analyzed the primary proliferative response induced in vitro in PBMC from HIV-1-seronegative donors by individual peptides covering the HIV-1 p24 protein sequence and shown that peptide 271-290 was able to elicit proliferation (6, 65). Our approach was different in that we used peptides covering the entire p55 protein that we pooled to perform in vitro immunization.

The HIV-1 Gag peptide 480-500 (P49), corresponding to the C terminus of p6, which we identified as the epitope of the clone 74 is a novel CD4$^+$ T helper epitope not reported previously. Earlier studies performed on PBMC from HIV-1-seronegative donors did not include peptides outside p24, and the studies on PBMC from HIV-1-seropositive individuals did not identify this peptide as a determinant of the CD4 response against HIV-1. This region of Gag is not very conserved and is truncated in some isolates of HIV-1.

The immune response to the HIV-1 Gag peptides that we observed in vitro may correspond to the immunogenicity that these peptides exhibit in vivo. For example, it has been shown that the in vitro response of T cells to varicella-zoster virus peptides is concordant with the response induced in vivo after immunization with varicella-zoster vaccine (23) and that a peptide identified in vitro as the primary T-cell epitope of *Chlamydia trachomatis* induces cell-mediated immunity and partial protection in vivo (26). Furthermore, two studies have analyzed the in vivo T helper immune response in macaques and in humans immunized with p24-bearing virus-like particles and found reactivity to peptides homologous to the ones reported in this study (35, 41, 66).

Further indication that some of the peptides identified here may be effectively presented during HIV-1 infection is provided by the observation that the epitopes of clones 6 and 74 are indeed presented when Gag is expressed with a recombinant vaccinia virus. The failure of the other clones to be activated by recombinant vaccinia virus-infected cells or by p24 protein raises the possibility that these cells would not be activated in vivo during HIV infection. However, as discussed in Results, this result may also reflect the limitations of the in vitro system used. Moreover, the fact that some of the peptides we identified as CD4$^+$ T-cell epitopes have been shown by others to be recognized by CD4$^+$ T cells from HIV-1-positive patients suggests that these peptides may be effectively presented through the natural processing of HIV-1 Gag during infection.

Peptides 278-292 and 265-286 were found to induce proliferative responses in 50 and 33%, respectively, of HIV-1-positive individuals (2, 67). Peptides almost equivalent to our immunodominant peptide 271-290 and to peptide 241-260 were identified by Rosenberg et al. among the determinants of the strong p24-specific helper cell response observed in two long-term nonprogressor HIV-1-positive patients (55). Furthermore, in a recent study, the latter group isolated clones from HIV-1-infected individuals and characterized epitopes that overlapped the epitopes found in our study, in particular in the region between amino acids 231 and 292 of Gag (44). These results collectively suggest that this region is one of the most immunogenic regions of HIV-1 Gag. The region is able to induce a CD4$^+$ T-cell response by immunization following natural HIV-1 infection, by vaccination of HIV-1-negative individuals with p24, and following in vitro stimulation of PBMC from HIV-1-negative individuals.

Based on their ability to induce an HIV-1-specific immune response, irrespective of whether this is a primary or secondary response, some of the peptides that we identified could be candidates to include in a vaccine against HIV-1. Due to the polymorphic nature of HLA, only if a peptide could be accommodated in the groove of several HLA class II molecules would it seem a suitable vaccine candidate (14). The HLA class II type of the donor we used was DR4, DR7, DQ2, and DQ3, and we showed that all the peptides that we identified were recognized by CD4$^+$ T cells in the context of HLA DR (Fig. 7). Each of the peptides contained a putative HLA DR4 or DR7 binding motif, based on data reported by Marsh et al. and Hammer et al. (16, 17, 34). Two possible binding motifs were found in peptide 271-290. Of note, as only one individual was used in our study, it is not possible to determine whether the capacity to mount an in vitro primary immune response to Gag peptides is related to HLA class II type. In earlier similar studies, the HLA types of the donors were not specified.

Functional analysis of the in vitro-generated HIV-1 Gag- and Gag peptide-specific CD4$^+$ T-cell clones indicates that most of the clones proliferate to peptide concentrations that fall within the range of those reported in the literature from immune donors, showing that the clones that we obtained after in vitro immunization have binding characteristics similar to those of clones raised in vivo (1, 12, 20, 44, 57, 64). Both Th1 and Th2 types of CD4$^+$ T cells were obtained, suggesting that by immunizing with HIV-1 Gag peptides, it may be possible to induce a CD4$^+$ T-helper response of both types in PBMC from a seronegative individual. The presence of CD4$^+$ T helper cells that could support both the cellular (Th1) and humoral (Th2) responses against HIV-1 could be crucial for protection from HIV-1 infection. Hasenkrug et al. demonstrated the requirement for B cells, CD4$^+$ T cells, and CD8$^+$ T cells in protection from Friend virus infection in a mouse model (19).

Similarly, multiple components of the immune response are likely to be important in the control of HIV-1 replication and spread. As noted above, the main role of CD4$^+$ T cells may reside in their ability to maintain functional cytotoxic CD8$^+$ T-cell responses. The mechanism by which T helper cells fulfill this role is poorly understood. A role for IL-2, IL-7, IL-15, and more recently IL-4 has been evoked, although more for the development than for the maintenance of the CD8 response (9, 33, 58). Such role in HIV infection remains to be demonstrated, and it is thus unclear whether the CD4$^+$ T-cell clones that we have characterized could fulfill this role.

As well as their helper role, CD4$^+$ T cells can also have a direct antigen-specific cytotoxic activity, as shown here by the activity of clone 6 against vaccinia virus Gag-infected cells. Interestingly, Gag-specific CD4$^+$ T-cell clones isolated by Norris et al. from HIV-1-infected individuals also displayed cytotoxic activity (44). Even though the function of CD4$^+$ T cells as killer cells is usually not considered as important as their role as helper cells (45), this function may still contribute to the elimination of HIV-1-infected cells and could play a role in vivo in the control of HIV-1 infection by CD4$^+$ T cells.

In summary, the results presented provide evidence that HIV-1 Gag peptides are able to induce a strong CD4$^+$ T-cell immune response in PBMC from an HIV-1-seronegative donor in vitro. The clones isolated represent a useful tool for the analysis of interactions between CD4$^+$ and CD8$^+$ T cells in vitro and in the hu-PBL-SCID mouse model of HIV-1 infection. The immunodominant peptides that were identified, in particular the peptides corresponding to the conserved region of HIV-1 Gag between amino acids 231 and 292, may represent potential candidates to be included in a vaccine against HIV-1. Finally, some of the clones that we have identified are in many aspects similar to clones isolated from HIV-1-infected patients in a recent study by Norris et al.; they recognize the same region of the Gag protein, produce IFN-γ, and display cytotoxic activity. This may indicate that some of the clones that we have characterized are representative of clones raised in vivo during the natural infection. However, we also note that the opposite argument can be made. The similarity between the CD4$^+$ T-cell responses in seropositive and seronegative individuals suggests that studies of the CD4$^+$ T-cell response in HIV-infected persons, especially with prolonged in vitro antigenic stimulation, could be reflecting in vitro-primed or cross-reactive responses and, as suggested by Norris et al. in their discussion, not responses that exist in vivo. Our study certainly raises concerns about this point and suggests that care should be taken in the methods used to study the CD4$^+$ response in HIV-infected patients.

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